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The Association of Blood Serum Atherogenicity with Risk Factors for Cardiovascular Disease

[1,2]Tatiana Vladimirovna Kirichenko, [1,2]Igor Alexandrovich Sobenin, [3]Veronika Alexandrovna Myasoedova, [2]Andrey Vladimirovich Omelchenko, [4]Sergey Gennadyevich Kozlov and [2,5]Alexander Nikolaevich Orekhov

[1]Laboratory of Medical Genetics, Chazov National Medical Research Center of Cardiology, Ac. Chazov Str. 15A, Moscow, Russia
[2]Laboratory of Angiopathology, Institute of General Pathology and Pathophysiology, Baltiyskaya Str. 8, Moscow, Russia
[3]Centro Cardiologico Monzino IRCCS, Str. Carlo Parea 4, Milan, Italy
[4]Department of Atherosclerosis Problems, Chazov National Medical Research Center of Cardiology, Ac. Chazov Str. 15A, Moscow, Russia
[5]Department of Atherosclerosis, Institute for Atherosclerosis Research, Str. Osennyaya 4, Moscow, Russia

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Corresponding Author:
Tatiana Vladimirovna Kirichenko
Laboratory of Medical Genetics, Chazov National Medical Research Center of Cardiology, Ac. Chazov Str. 15A, Moscow, Russia
Email: t-gorchakova@mail.ru

Abstract: Cardiovascular Diseases (CVD) caused by atherosclerosis are the most frequent cause of mortality worldwide, so the identification of new biomarkers of atherosclerosis development is quite actual. The atherogenicity of blood serum is considered as the potential of human serum to cause cholesterol accumulation in primary culture macrophages. The current study aimed to evaluate the association of the atherogenicity of blood serum with conventional cardiovascular risk factors to assess its potential in CVD prognosis. Serum atherogenicity was measured in 815 study CVD-free participants at baseline and at the follow-up visit after 5 years. 51% of study participants had atherogenic serum at baseline, but at the follow-up visit after 5 years atherogenicity increased significantly in 60% of participants. An increase of atherogenicity was observed in the group with non-atherogenic serum at baseline, $p<0.001$. The correlation of serum atherogenicity with plasma lipids level, i.e., total cholesterol and LDL, was demonstrated. There was a negative correlation between changes in the potential of blood serum to induce cholesterol accumulation in primary culture macrophages and the age of study participants ($r = -0.089$, $p = 0.011$). In groups with decreased and unchanged atherogenicity, the increase of statin administration, as well as amelioration of lipids profile, were revealed after a 5-year follow-up. Thus, the atherogenicity of blood serum may be considered a promising marker in the prognosis of CVD development, but further research is needed to evaluate the prognostic value of serum atherogenicity.

Keywords: Atherosclerosis, Serum Atherogenicity, Intracellular Cholesterol Accumulation, Cardiovascular Risk Factors

Introduction

Nowadays numerous instrumental methods are used for the diagnosis of atherosclerosis and the prognosis of its development, which allows characterizing atherosclerotic changes in the arterial wall qualitatively and quantitatively; a comprehensive analysis of multiple biomarkers and cardiovascular risk factors is conducted to identify patients with high risk of atherosclerosis. However, Cardiovascular Diseases (CVD) caused by atherosclerosis still remain the most frequent cause of mortality worldwide (Afzal, 2021). In this regard, the identification of new biomarkers and instrumental methods for the long-term prognosis of atherosclerosis is an urgent task for modern science. Regular screening is necessary for early detection of atherosclerosis, evaluation of the treatment effectiveness, and prognosis. Ultrasound investigation of carotid arteries based on an assessment of carotid plaque burden, vessel wall volume, and Intima-Media Thickness (cIMT) is an effective method of non-invasive imaging of atherosclerotic lesions (Amato *et al*., 2017; Spence, 2023). Coronary computed tomography angiography allows determining parameters used for the prognosis of atherosclerosis progression in coronary arteries such as plaque volume and burden (Feng *et al*., 2023). Numerous studies devoted to the analysis of genetic predisposition to

the development and progression of atherosclerosis have identified loci associated with carotid atherosclerosis, atherosclerosis of peripheral arteries, and atherosclerosis-associated diseases (Yeung *et al*., 2022). Atherosclerosis is a multi-factorial disease, that includes different pathogenetic processes including oxidative modification of the lipoproteins and endothelial dysfunction leading to recruitment, migration, and differentiation of immune cells in the arterial wall, which is accompanied by the production of inflammatory mediators that cause the development of inflammatory reactions in the arterial wall and pro-inflammatory polarization of macrophages, that is an important step in the formation of atherosclerotic plaques (Morrison *et al*., 2023; Tabas and Bornfeldt, 2016; Libby, 2021). The key initial stage of atherosclerosis development is the intracellular accumulation of cholesterol in the arterial wall that leads to the foam cells formation (Li *et al*., 2021). The atherogenicity of blood serum is considered as the potential of human blood serum to cause the accumulation of cholesterol in the primary culture of macrophages (Orekhov *et al*., 1988). It was demonstrated that the blood serum of patients with atherosclerosis of coronary arteries had atherogenic potential in contrast to the blood serum of patients without coronary artery atherosclerosis (Orekhov *et al*., 1988; Tertov *et al*., 1989). In this regard, the use of a cellular test that allows determining the potential of blood serum to cause cholesterol accumulation in the primary culture of macrophages looks interesting and promising in terms of applicability as an *in vitro* test for CVD risk assessment (Myasoedova *et al*., 2017). Until now, long-term changes in blood serum atherogenicity haven't been studied, so the use of the test is quite limited. The open-label prospective study was conducted to investigate the dynamics of changes in blood serum pro-atherogenic properties over a long period of observation and its association with conventional cardiovascular risk factors.

Materials and Methods

Patients

The study was conducted according to the principles regulated by the Declaration of Helsinki. The study protocol and documentation were approved by the Committee on Ethics of the Institute for Atherosclerosis Research, Moscow (protocol of Ethics Committee #038-15 of December 2, 2015). Each study participant received a study information sheet prior to signing the informed consent. The study participants signed the informed consent for the participation in the study. The rights of study participants for privacy and confidentiality of personal data were protected. The study participants had the right to be excluded from the study on their own at any moment without giving a reason. The study was population-based, the prognostic value of blood serum atherogenicity was assessed in complex with conventional cardiovascular risk factors. Inclusion criteria were age over 40 years and absence of CVD. Persons meeting the inclusion criteria were enrolled in the study during the regular medical examination. The following conventional cardiovascular risk factors were evaluated in the study population: Arterial blood pressure, Body Mass Index (BMI), smoking, family history of myocardial infarction, presence of type-2 diabetes, and lipids profile. The 10-year cardiovascular risk of study participants was determined using the Framingham risk score (Agostino *et al*., 2008). Measurements of cIMT by B-mode ultrasound were used to characterize the predisposition to atherosclerosis in carotid arteries (Touboul *et al*., 2012). Coronary calcification was evaluated by Computed Tomography (CT). The Coronary Artery Calcium (CAC) score was evaluated using the Agatston method (Agatston *et al*., 1990). The blood lipid profile included the assessment of total cholesterol, Triglycerides (TG), High-Density Lipoproteins (HDL), and Low-Density Lipoproteins (LDL). Laboratory diagnostics also included C-reactive protein (CRP) measurement.

In total, 815 participants (420 females, and 395 males) were included in the study. The study participants were free of CVD; the mean age of study participants was 59.2 [7.9] years old. Most of the study participants were overweight, and 20% had diabetes mellitus. Most of the study participants had disorders in the blood lipid profile-the increase of total cholesterol and LDL.

Blood Serum Atherogenicity

The investigation of atherogenicity of blood serum was performed in a cell culture-based assay by measurement of the cholesterol content in the primary culture of macrophages after incubation with the blood serum of study participants. Monocytes were isolated from 50 mL of venous blood of healthy volunteers by centrifugation in the medium for lymphocyte separation LSM 1077. Then isolated cells were planted into a cell culture plate and were incubated for 24 h in Dulbecco's Modification of Eagle's Medium (DMEM) with the addition of fetal calf serum at a 10% concentration. After 24 h of cultivation, the culture medium was refreshed and cells were cultivated in DMEM with the addition of a tested serum sample at a 10% concentration. All tests were performed in triplicates in a single well for each patient. The samples of blood serum of each participant obtained at baseline and at the follow-up visit after 5 years were analyzed in a single experiment to allow valid comparison of samples. Before the start of the experiments, 2 samples of pooled sera were prepared based on the potential to cause cholesterol accumulation

in cultured cells. The first sample, which did not induce cholesterol accumulation was used as a negative control and the 1 sec, which induced an increase in cholesterol cellular content by 1.34 (SD 0.06) fold, was used as a positive control. Both control samples were used in all experiments to reduce the between-culture variability of results. Additionally, a sample of pooled serum with previously assessed atherogenic potential was used in all experiments to obtain a reference value. All cellular tests were performed in a blinded manner, meaning the researchers did not have access to the patient's clinical data.

Cholesterol Extraction and Measurement

The cells were incubated with tested serum for 3.5 h and then were washed with Phosphate-Buffered Saline (PBS) (Sigma-Aldrich, USA) and fixed with a mixture of hexane and isopropanol 1:1. The lipids were extracted from the fixed cells with hexane-isopropanol mixture (Hara and Radin, 1978). The lipid extracts were evaporated in microplates at room temperature under airflow. The dried extracts were resolved in a mixture of PBS, isopropanol, sodium cholate, and Triton X-100. Cholesterol concentration was measured by enzyme assay using commercial kits Fluitest CHOL performed with colorimetry method (Bio Tek Instruments, USA). Calibration was performed with a standard cholesterol solution at a concentration of 0.5 mg/mL. Delipidated cells were resolved in a solution of sodium hydrate for the assessment of the protein content performed with the Lowry method (Waterborg and Matthews, 1984). Standard bovine serum albumin solution at a concentration of 10 mg/mL (Thermo Fisher Scientific, USA) was used for calibration. The cholesterol value in each well was divided by the protein value in the same well and was presented as μg/mg of protein content. Cholesterol accumulation in cells incubated with a sample of pooled non-atherogenic serum used as a negative control was set for 100%. The blood serum of each study participant was defined as atherogenic if induced statistically significant increase of intracellular cholesterol accumulation in comparison with control experiments. Blood serum was defined as non-atherogenic if induced no statistically significant change in intrace cholesterol accumulation in comparison with control. Quantitative estimation of serum atherogenicity was based on the measurement of intracellular cholesterol content compared to that of control cells, taken for 100%.

Statistical Analysis

The sample size was estimated based on Cochran's equation, based on a precision level of 5%, a confidence Interval of 95%, and a maximum level of variability in the compared samples (estimated proportion) of 0.5. The required size of each sample was 385 patients. The results were presented as mean value and Standard Deviation (SD). Statistical program package SPSS 27.0 (SPSS Inc., USA) was for analysis of the study results (IBM, 2021). The difference between groups was assessed by t-test and Mann-Whitney analysis. The changes in parameters after the follow-up period were analyzed by Wilcoxon analysis. Correlation analysis and regression analysis were conducted to identify cardiovascular risk factors associated with serum atherogenicity dynamics.

Results

Serum Atherogenicity of Study Participants

The serum atherogenic potential of study participants were analyzed at baseline and after 5 years of follow-up. The observed population was non-homogeneous for the initial levels of blood atherogenicity; therefore, the study participants were divided into 2 groups: Non-atherogenic and atherogenic blood serum. At baseline, the blood serum of 398 study participants (205 females, 193 males) didn't induce significant cholesterol accumulation in the primary culture of macrophages, i.e., wasn't atherogenic. Blood serum of 417 study participants (215 females, 202 males) induced significant intracellular cholesterol accumulation, i.e., was atherogenic at baseline. The prevalence of serum atherogenicity among the study participants is presented in Table 1.

In group 1 with non-atherogenic blood serum, the initial value of the cholesterol accumulation was 103.4 (9.4). In group 2 with atherogenic serum at baseline, this parameter was 151.3 (74.4). After 5 years of follow-up, there was a statistically significant increase of serum atherogenicity in group 1 up to 117.8(27.9), $p<0.001$. By contrast, in group 2, serum atherogenicity decreased to 147.3 (65.3), $p = 0.058$. The atherogenicity of blood serum wasn’t significantly different between men and women at baseline and after 5 years of follow-up.

Table 1: Serum atherogenicity prevalence

Serum atherogenicity	Non-atherogenic group	Atherogenic group	Difference between groups, p
Baseline, %	103 (9)	151 (74)	<0.001*
Follow-up, %	118 (28)	147 (65)	<0.001*
Significance of change, p	<0.001*	0.051	

Table 2: Baseline clinical and laboratory characteristics of study groups

Characteristics	Non-atherogenic n = 398	Atherogenic n = 417	Significance of difference, p
Age, years	59.1 (7.7)	59.4 (7.7)	0.578
Male sex, %	48.0	48.0	0.988
BMI, kg/m^2	28.0 (4.7)	28.4 (4.7)	0.239
Smoking, %	59.0	54.0	0.194
SBP, mm Hg	133 (21)	132 (21)	0.671
DBP, mm Hg	81 (12)	80 (11)	0.330
Diabetes, %	20	19	0.612
CAC, Agatston score	200 (560)	205 (649)	0.899
Risk of CVD, Framingham score	11.1 (7.3)	11.5 (8.4)	0.435
cIMT, mm	0.680 (0.127)	0.689 (0.148)	0.356
Family history of MI, %	31	26	0.148
Statin administration, %	12	12	0.730
CRP, mg/dl	0.38 (1.12)	0.37 (2.27)	0.121
Total cholesterol, mg/dl	225.9 (36.3)	232.0 (38.2)	0.019*
HDL, mg/dl	57.6 (16.3)	58.7 (17.0)	0.327
LDL, mg/dl	146.8 (36.0)	150.4 (35.9)	0.159
TG, mg/dl	142.3 (99.0)	148.8 (97.2)	0.340

Data presented as mean (SD)
BMI, body mass index; SBP, systolic blood pressure; Diastolic Blood Pressure, (DBP); CAC, coronary artery calcium; cIMT, intima-media thickness of carotid arteries; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides

Clinical Characteristics of Study Participants

Baseline clinical and laboratory characteristics of study participants are presented in Table 2. The age of the participants varied from 46-77, mean age was 59.1 (7.7) in the non-atherogenic group and 59.4 (7.7) in the atherogenic group. Study participants had increased BMI but were not obese. Some of the participants (20% of participants with non-atherogenic serum, and 19% of participants with atherogenic serum) had type 2 diabetes. Most of the participants had normal blood levels of total cholesterol. The groups were not significantly different in terms of conventional cardiovascular risk factors such as age, gender, BMI, smoking status, blood pressure, family history of myocardial infarction, diabetes, cardiovascular risk calculated with Framingham score and blood lipids profile except for total cholesterol level, which was higher in the group with atherogenic serum ($p = 0.019$). The groups also were not significantly different in terms of statin intake, coronary calcification calculated with Agatston score, CRP level, and cIMT.

All study participants underwent the second clinical and laboratory examination after 5 years. Table 3 presents the characteristics of study groups after a 5-year follow-up period. After a 5-year follow-up period, coronary artery calcium content increased significantly in both groups. Statins administration increased significantly in both groups, at the same time, clinically insignificant but statistically significant improvement of all lipid profile parameters was determined in both groups after 5 years of follow-up.

Association of Serum Atherogenicity with Cardiovascular Risk Factors

Pearson correlation analysis was conducted to study the association of serum atherogenicity with the clinical and laboratory parameters of study participants. No correlation of serum atherogenicity with any clinical or laboratory characteristic was observed in the total group. The negative correlation of the serum atherogenicity change with age of study participants was revealed $r = -0.084$, $p = 0.011$). In the group with non-atherogenic serum, the correlation of serum atherogenicity with total cholesterol ($r = 0.115$, $p = 0.023$) and triglycerides ($r = 0.119$, $p = 0.018$) was observed. After 5 years of follow-up, serum atherogenicity correlated with LDL level ($r = 0.112$, $p = 0.026$) in this group.

Regression analysis was conducted to identify risk factors for CVD associated with serum atherogenicity dynamics. Relative change of serum atherogenicity (fold) was used as a dependent variable, while observed risk factors for CVD were used as independent variables. A high proportion of the variability of the dependent variable was shown in the resulting linear regression model ($R2 = 0.939$; $F = 1235.663$; $p<0.001$). According to this model, the following factors determined the increase of serum atherogenicity (in descending order of significance): Baseline atherogenicity, total cholesterol level, relative changes of HDL and LDL, CVD risk by Framingham risk score, systolic blood pressure, age of study participants, BMI, TG level and smoking.

Table 3: Characteristics of study groups after a follow-up period

	Non-atherogenic group		Atherogenic group	
Characteristics	Follow-up results	Significance of change, p	Follow-up results	Significance of change, p
BMI, kg/m^2	28.4 (4.9)	<0.001*	28.7 (4.9)	<0.001*
SBP, mm Hg	132 (19)	0.258	131 (20)	0.001*
DBP, mm Hg	80 (11)	0.001*	79 (11)	0.005*
CAC, Agatston score	327 (692)	<0.001*	300 (876)	<0.001*
Total cholesterol, mg/dl	221.0 (38.3)	0.007*	222.0 (44.7)	<0.001*
HDL, mg/dl	59.1 (14.3)	0.011*	59.8 (16.0)	0.026*
LDL, mg/dl	137.9 (33.3)	<0.001*	136.2 (35.9)	<0.001*
TG, mg/dl	132.3 (82.1)	0.018*	135.2 (81.0)	0.002*
CRP, mg/dl	0.28 (0.36)	0.063	0.29 (0.59)	0.480
Statin administration, %	17	0.002*	22	<0.001*

Data presented as mean (SD)
BMI, body mass index; SBP, systolic blood pressure; Diastolic Blood Pressure, (DBP); CAC, coronary artery calcium; cIMT, intima-media thickness of carotid arteries; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides

Further, the total group was divided into the groups of study participants with increased serum atherogenicity after 5 years of follow, (n = 156), with unchanged serum atherogenicity, (n = 569), and with decreased serum atherogenicity, (n = 90). The significant difference in changes in clinical and laboratory parameters during the follow-up period between groups was observed in terms of lipid profile and statin administration. It was demonstrated that statin administration increased significantly in groups with decreased (n = 89) and unchanged (n = 560) atherogenicity from 15-28%, n = 0.004 and from 12-21%, p<0.001, respectively as well as the significant amelioration of lipids profile was observed in these groups. In the group with increased atherogenicity, the statin administration didn't change significantly and was 12% at baseline vs. 13% in 5 years, p = 0.594, blood lipid level also didn’t change in this group, except LDL reduction from 148 (37)-140 (36), p = 0.001.

Discussion

In this study, the association of intracellular cholesterol accumulation in cell culture under the influence of study participants' blood serum with conventional cardiovascular risk factors was investigated. The atherogenicity of blood serum was first described in a sufficiently large population of more than 800 participants aged 45-75 without signs of clinical manifestation of atherosclerosis. Atherogenic serum-induced intracellular accumulation of lipids in a cellular test that was significantly different in comparison with control cells. At baseline, blood serum had atherogenic potential only in half of the surveyed patients. After the follow-up period, serum atherogenicity increased significantly in the group of participants who had non-atherogenic serum at baseline. In the group of participants who had atherogenic serum at baseline, the potential of blood serum to cause cholesterol accumulation in the primary culture of macrophages decreased but didn't differ significantly after 5 years of follow-up. A significant negative correlation between the changes in serum atherogenicity and age of participants was revealed.

Previously in the other study, the correlation between atherogenicity of blood serum and CAC was analyzed (Sobenin *et al*., 2014). The study demonstrated the significant increase in atherogenicity of blood serum after the observation period, as well as the significant increase of CAC investigated with the use of Agatston CAC score. However, no correlation between the changes in atherogenicity of blood serum with CAC dynamics was observed. Probably absence of correlation is due to the fact that coronary calcification reflects the progression of moderate atherosclerotic lesions while the atherogenicity of blood serum induces one of the first steps of atherosclerosis development such as cholesterol accumulation. In the other study, the association of serum atherogenicity with cIMT was demonstrated in 196 men with subclinical atherosclerosis (Kirichenko *et al*., 2016).

The results of the study demonstrate that the probability of serum atherogenicity increase was higher in younger participants. The possible reason for this fact may be the non-linear, but power-shaped or sigmoidal increase of an individual's serum potential to induce cholesterol accumulation in macrophages with aging. So, younger participants were included in the study at the time when atherogenicity started rising from individual baseline levels, whereas older participants were included when atherogenic potential had already reached its maximum. However, this hypothesis could not be tested in the study, since the most important limitation of this study was the fact that no interim visits were held.

Previously cellular tests weren't performed for evaluation of serum atherogenicity in long-term observational studies. In numerous studies the atherogenicity of blood serum is measured based on blood lipid parameters and age-related changes in atherogenicity index were demonstrated. In particular, it was shown that blood levels of total cholesterol, LDL, and triglyceride as well as plasma atherogenic index decreased with age in patients with ischemic heart disease (Hong *et al*., 2022). The other study of the association between plasma atherogenic index and risk of myocardial infarction demonstrated that a high plasma atherogenic index calculated as a ratio of blood triglycerides and HDL led to a greater risk of myocardial infarction in younger patients (Zhang *et al*., 2023).

Currently, cellular cultures are widely used for the assessment of cholesterol accumulation in different experimental studies in the field of atherogenesis and cardiovascular health. In particular, the atherogenic potential of amino acids was studied in the macrophage culture where cellular toxicity, reactive oxygen species production, and intracellular cholesterol or triglyceride content were assessed in macrophages cultured with increasing concentrations of anti-or pro-atherogenic amino acids (Rom *et al*., 2017). Test systems based on this principle are used in multiple studies for the evaluation of the efficacy of agents targeting the amelioration of atherosclerosis development (Zhang *et al*., 2020; Kavitha *et al*., 2019; Karten *et al*., 2021). Experimental studies are currently the most appropriate way to use serum atherogenicity as one of the endpoints in assessing the effectiveness of anti-atherosclerotic preparations since it reflects the major initial step of atherosclerosis development such as intracellular cholesterol accumulation. In our previous studies, significant changes in serum atherogenicity in clinical trials of statins and calcium antagonists, natural preparations based on garlic and phytoestrogens were demonstrated (Kirichenko *et al*., 2017; Orekhov *et al*., 1997). The present long-term study in a sufficiently large sample was aimed to assess the association of serum atherogenicity with conventional risk factors for CVD, however, there is still not enough clinical data on the diagnostic value of blood serum atherogenicity to use this test as a prognostic marker in epidemiologic research.

Conclusion

The results obtained in the current study show that serum atherogenicity is a potential mechanistic marker of atherosclerosis. Additional research is needed for a more in-depth study of the association of the atherogenicity of blood serum with the parameters of subclinical atherosclerosis, namely with the progression of IMT of carotid arteries, as well as with risk factors and signs of clinical manifestations of atherosclerosis to determine the significance of this indicator in long-term predicting of atherosclerosis development.

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Author's Contributions

Tatiana Vladimirovna Kirichenko: Coordination of the data analysis and written of the manuscript.

Igor Alexandrovich Sobenin: Development of methodology and study design, contribution to the written of the manuscript.

Veronika Alexandrovna Myasoedova: Participation in all experiments and data curation.

Andrey Vladimirovich Omelchenko: Coordination of the study and statistical analysis of study results.

Sergey Gennadyevich Kozlov: Participation in all experiments and coordination of the study.

Alexander Nikolaevich Orekhov: Design and organization of the study, contribution to the written of the manuscript.

Ethics

All authors approved the manuscript and declared no ethical issues as well as no conflict of interest. This manuscript is original and reports unpublished data.

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